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(PATENT)

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IN RE APPLICATION OF:
Iwao KATSUYAMA et al.

Application No.: 10/526,369

Filed: March 3, 2005

For: METHOD OF SCREENING PHYSIOLOGICALLY ACTIVE SUBSTANCE

Confirmation No.: 6217

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EXAMINER: Ogunbiyi, Oluwatosin A,

DECLARATION UNDER 37 C.F.R. 1.132

Commissioner for Patents
P.B. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Masao Tokunaga, Ph.D., residing in Kagoshima-ken, Japan, hereby declares and states as follows:

1. That I am one of the co-inventors of U.S. Application Serial No. 10/526,369 filed on March 3, 2005, entitled METHOD OF SCREENING PHYSIOLOGICALLY ACTIVE SUBSTANCE. I am thoroughly familiar with the contents of said Application, its prosecution before the United States Patent and Trademark Office and the references cited therein.

2. That I am a graduate of Graduate School of Osaka Prefecture University, Department of Agriculture, received a doctorate in the year 1976, majoring in agricultural chemistry.

3. That I have been extensively engaged in the field of biotechnology for at least 30 years, and is a professor at the Laboratory of Applied and

Molecular Microbiology, Faculty of Agriculture, Kagoshima University since 1993; my curriculum is attached as Appendix I hereto.

4. That the following experiments were conducted under my supervision and control in order to verify that transformants maintain a given growth rate in the respiration ability-deficient strains, but such properties cannot be secured in the wild-type strains.

EXPERIMENTAL METHODS AND EXPERIMENTAL RESULTS

Strains, media, and reagents used in the present experiment, experimental methods and experimental results are summarized as follows:

1. Strains

Strains used in the present experiment are shown in the following table.

Table

Microorganism	Strain	Genotypes
E. coli	JM109	*hsd*R17(rK$^-$,mK$^+$), *rec*A1, *end*A1, *Δ(lac-pro*AB), *gyr*A96, *thi*-1, *rel*A1, *sup*E44, F'[*tra*D36, *pro*AB, *lac*I^q, *lacZΔ*M15], e14$^-$(*Mcr*A$^-$)
S. cerevisiae	YPH500	*MATα*, *ura3-52*, *lys2-801*^{amber}, *ade2-101*^{ochre}, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*
S. cerevisiae	YPH500-12	*MATα*, *ura3-52*, *lys2-801*^{amber}, *ade2-101*^{ochre}, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*, ρ^0 (or ρ^-)

2. Media and Reagents

In the present experiment, media and reagents described in *Laboratory Course Manual for Methods in Yeast Genetics, 2000 Edition*, authored by Burke, D., Dawson, D., and Stearns, T., Cold Spring Harbor Laboratory Press (2000) were used for each experiment. All of the media were autoclaved and used.

In addition, NU1025 (8-hydroxy-2-methylquinazoline-4-one), a selective inhibitor for PARP, is one manufactured by CALBIOCHEM.

3. Experimental Methods

(1) Transformation of *Escherichia coli* JM109

One-hundred microliters of competent cells of JM109 stored at -80°C were thawed on ice, and 5 μL of plasmid DNA was added thereto, and the mixture was allowed to stand on ice for 30 minutes. After the mixture was subjected to heat shock at 42°C for 30 seconds, the resulting mixture was allowed to stand for 2 minutes on ice, and 0.9 mL of a SOC medium was added thereto. The components were thoroughly mixed, and the cells were cultured at 37°C for 45 minutes. The resulting culture was plated on an LB + Amp solid medium having an Amp concentration of 100 μg/mL, and the cells were cultured overnight at 37°C.

(2) Method of Preparing Medium Scale of Plasmid DNA (Medium-Scale Extraction)

Cells previously cultured in 30 mL of the LB + Amp liquid medium were transferred to a centrifugation tube, and the cells were harvested by centrifuging the culture at room temperature for 5 minutes at 8000 r/min. To the precipitates was added 3 mL of a lysozyme solution (5 mg lysozyme/1 mL glucose buffer) to gently lyse the precipitates, and the mixture was allowed to stand on ice for 5 minutes. Thereto was added 4 mL of an NaOH-SDS solution (0.2 N NaOH - 1% SDS) while gently mixing, and the mixture was allowed to stand on ice for 5 minutes. Thereto was added 3 mL of a 3 M sodium acetate solution (pH 4.8)

while gently mixing, and the mixture allowed to stand on ice for 10 minutes, and centrifuged at 4°C for 15 minutes at 15,000 r/min. The supernatant was transferred to a fresh tube, and 6 mL of isopropanol was added thereto. The mixture was allowed to stand at -20°C for 20 minutes, and centrifuged at 4°C for 15 minutes at 15,000 r/min. The precipitates were washed with 2 mL of 70% ethanol, dissolved in 300 μL of TE, and transferred to a microfuge tube. The centrifugation tube was similarly washed with 300 μL of TE, and the TE was transferred to the microfuge tube (total of 600 μL). Thereto was added 6 μL of 10 mg/mL RNase, and the mixture was incubated at 37°C for 20 minutes. The mixture was extracted three times with phenol, extracted three times with chloroform, and extracted three times with ether. To the resulting extract were added 120 μL of a 5 M NaCl solution and 240 μL of 30% PEG #6000 while mixing, and the mixture was allowed to stand at -20°C for 40 minutes, and allowed to stand at 4°C for 30 minutes. After the mixture was centrifuged at 4°C for 10 minutes at 14,000 r/min, the precipitates were harvested with complete removal of water from the tube, and dissolved in 80 μL of sterile water. Thereto were added 4.8 μL of a 5 M NaCl solution and 192 μL of ethanol while mixing, and the mixture was allowed to stand at -20°C for 30 minutes. After the mixture was centrifuged at 4°C for 15 minutes at 14,000 r/min, 200 μL of 70% ethanol was added to the precipitates to gently wash the precipitates. After the mixture was centrifuged at 4°C for 15 minutes at 14,000 r/min, the precipitates were dried with a suction pump, and dissolved in 100 μL of TE. Thereafter, its concentration was determined by an OD_{260} value and electrophoresis.

(3) PCR Method

PCR was performed for 25 cycles under the conditions that one cycle consisted of 98°C for 30 seconds, a given temperature between 55° to 72°C for 60 seconds and 72°C for 90 seconds. The reaction was performed by adding 5 μL each of 50 μM primers, 10 μL of NEB × 10 buffer, 10 μL of a 4 mM 4dNTP mix, 0.5 μL of NEB Ventpol (DNA polymerase) (2U/μL) and 59.5 μL of sterile water, respectively, to 10 μL of a 5 ng/μL sample DNA to adjust its volume to a total of 100 μL, and adding 1 μL of 10 mg/mL BSA and 60 μL of a mineral oil thereto.

(4) Isolation of DNA by Cut-out from Agarose Gel

The amount 5 μg of plasmid DNA prepared by the medium-scale extraction was treated with a restriction enzyme in a 100 μL system, and thereafter 20 μL of Loading Dye was added thereto so as to make up a volume of 120 μL. Three combs having a width of 6 mm of an equipment for agarose gel electrophoresis were wound with a cellophane tape so as to gather those three combs into one. A 120 μL of a sample was applied to a gel, and electrophoresed. After the termination of electrophoresis, a desired band was cut out with a cutter knife while irradiating a gel with a 365 nm long-wavelength DNA cut-out lamp. The collected gel was placed into a dialysis tube containing 250 μL of 0.5 × TBE, and the dialysis tube was set in equipment for agarose gel electrophoresis, and electrophoresis was carried out for 30 minutes in a usual direction, and thereafter for 30 seconds in a reverse direction. The 0.5 × TBE was collected, and extracted twice with phenol, and extracted three times with ether.

(5) Small-Scale Process for Preparing Plasmid DNA (Mini-prep)

A colony of transformed *Escherichia coli* was inoculated into a 1.2 mL LB + Amp liquid medium contained in a microfuge tube, and cultured overnight at 37°C. After the culture medium was centrifuged at room temperature for 1 minute at 12,000 r/min, 100 μL of a glucose buffer was added thereto, and the mixture was vortexed to completely suspend the precipitates. To the suspension was added 150 μL of an NaOH-SDS solution (0.2 N NaOH-1% SDS), the tube was shaken vertically to gently mix them, and thereafter the mixture was allowed to stand on ice for 5 minutes. 150 μL of a 3 M potassium acetate buffer was added thereto while mixing, and 500 μL of phenol was added thereto while mixing. Thereafter, the mixture was centrifuged at 4°C for 5 minutes at 12,000 r/min. The upper layer (about 500 μL being collected) was transferred to another tube, an equivolume (500 μL) of chloroform was added thereto, and the mixture was centrifuged at 4°C for 5 minutes at 12,000 r/min. The upper layer (about 400 μL being collected) was transferred to another tube, a twice the volume (800 μL) of ethanol was added thereto while thoroughly mixing, and the mixture was allowed to stand at -20°C for 30 minutes. Thereafter, the mixture was centrifuged at 4°C for 10 minutes at 14,000 r/min, and 200 μL of 70% ethanol was added to the precipitates while gently mixing. The mixture was centrifuged at 4°C for 5 minutes at 14,000 r/min, and the precipitates were dried with a suction pump, and then dissolved in 20 μL of RNase-containing TE (10 mg/mL RNase 1 μL-TE 20 μL), and incubated at 37°C for 30 minutes. Thereafter, the resulting plasmid DNA was treated with a restriction enzyme, and confirmed by electrophoresis.

(6) Transformation of Yeast (Lithium Acetate Method)

An yeast was inoculated into 5 mL of an YPD liquid medium, and the mixture was pre-cultured overnight at 30°C. One milliliter of the culture medium was inoculated into 20 mL of an YPD liquid medium, and the cells were cultured at 30°C for 4 to 6 hours until a KU (Klett unit) value was adjusted to around 60. The OD_{600} value of the culture medium was determined, and the amount of the culture medium was calculated from a found value so that the number of cells is 2×10^8 cells. The calculated culture medium was weighed, and transferred to a 50 mL centrifugation tube. After the culture medium was centrifuged at room temperature for 5 minutes at 3,000 r/min, the precipitates were suspended in 10 mL of TE, and the suspension was similarly centrifuged at room temperature for 5 minutes at 3,000 r/min. The precipitates were suspended in 1 mL of TE, and 1 mL of a 0.2 M lithium acetate solution was added to the suspension. The mixture was cultured at 30°C for 1 hour (termination of the preparation of competent cells). Two-hundred microliters each of the prepared competent cells were dispensed into a 1.5 mL tube, 5 μg each of the DNA was added thereto, and the mixture was allowed to stand on ice for 30 minutes. Thereto was added 200 μL of 70% PEG 4000 while thoroughly mixing. The mixture was incubated at 30°C for 1 hour, and then subjected to heat shock at 42°C for 5 minutes. Thereafter, the mixture was immediately centrifuged at 4°C for 5 minutes at 12,000 r/min, and the precipitates were suspended in 1 mL of sterile water chilled on ice in advance. The suspension was centrifuged at 4°C for 5 minutes at 12,000 r/min. After the precipitates were suspended in 100 μL of chilled sterile water, the suspension was plated on a selected medium, and the cells were cultured at 30°C for 3 to 5 days. A KU value was determined with a

Klett Summerson photoelectric photometer (manufactured by Klett Manufacturing).

(7) Acquirement of Respiration Ability-Deficient Strain (ρ^-) of Yeast

An YPH500 strain (ρ^+) was transformed in accordance with a lithium acetate method, whereby Lyn-expressing plasmid pESC-TRP/Lyn was introduced. The resulting transformant was subcultured to a different CSM-TRP solid medium, and cultured at 30°C for 2 to 3 days, and thereby a transformant having no tryptophan-requiring property was screened. The screened transformant was inoculated into 5 mL of an YPD liquid medium, and the yeast was cultured until stationary growth was reached, and thereafter subcultured into 5 mL of a separate fresh YPD liquid medium and cultured. The yeast was cultured in the same manner for three times, and thereafter the culture medium was diluted 10^5 folds, and the dilution was plated on an YPD plate, and cultured for 2 to 3 days. Of the colonies grown on the YPD plate, a white colony was subcultured to a CSM complete medium (a medium containing all amino acids constituting the medium) and a CSM-TRP solid medium, and colonies from which the plasmid had been fallen away were screened. In other words, colonies that grow in the CSM complete medium, but do not grow in the CSM-TRP solid medium were screened. The screened colonies were subcultured into an YPD medium and an YPG medium (a medium containing glycerol as a carbon source), to confirm that the strain is a respiration ability-deficient strain (ρ^-). More specifically, it was confirmed that the colonies grow in the medium containing glucose as a carbon source (YPD medium) but do not grow in the medium

containing glycerol as a carbon source (YPG medium). The strain screened by the above procedures was named YPH500-12 strain.

(8) Expression of caf (*hcaf1*) Gene in Wild-Type Strain and Respiration Ability-Deficient Strain of Yeast *S. cerevisiae*

In this example, as a vector, pESC-URA was used for subcloning. In addition, as *hcaf1* gene, pcDNA3-*hcaf1* containing the gene (one in which *hcaf1*-myc gene was inserted into *Bam*HI-*Xho*I site of pcDNA3) was amplified, and the resulting gene was used.

(i) Preparation of pESC-URA/Caf1

Escherichia coli JM109 was transformed with pcDNA3-*hcaf1*, and plasmid DNA was prepared from the resulting transformant (medium-scale extraction). *Escherichia coli* was also transformed with pESC-URA, and plasmid DNA was prepared in the same manner (medium-scale extraction). The prepared plasmid pcDNA3-*hcaf1* was treated with *Bam*HI and *Xho*I, and DNA was collected by cut-out from a gel. Also, as to pESC-URA, the plasmid was treated with *Bam*HI and *Xho*I, and DNA was collected by cut-out from a gel. The ligation was carried out with *hcaf1* gene and a fragment of pESC-URA collected by these experimental procedures, and *Escherichia coli* was transformed with the resulting product. After the resulting transformant was confirmed by mini-prep, plasmid DNA was prepared (medium-scale extraction). A nucleotide sequence of the introduced gene was confirmed by sequencing from both sides of a gene-introduced portion.

(ii) Introduction of *hcaf1* Gene in YPH500 Strain (ρ^+) and YPH500-12 Strain (ρ^-)

An YPH500 strain (ρ^+) and an YPH500-12 Strain (ρ^-) were transformed with the prepared plasmid according to the lithium acetate method. The resulting transformant was subcultured to a different CSM-URA solid medium, and cultured at 30°C for 2 to 3 days, and thereby a transformant having no uracil-requiring property was screened.

(9) Expression of *lyn* Gene in Wild-Type Strain and Respiration Ability-Deficient Strain of Yeast *S. cerevisiae*

In this example, as a vector, pESC-TRP was used for subcloning. In addition, as *lyn* gene, pME-Lyn containing the gene (one in which *lyn* gene was inserted into *Mlu*I site of pME-SM [one having substitution of *Eco*RI site of pME18S with *Mlu*I site) was amplified, and the resulting gene was used.

(i) Preparation of pESC-TRP/Lyn

Escherichia coli JM109 was transformed with pME-Lyn, and plasmid DNA was prepared (medium-scale extraction) from the resulting transformant. *Escherichia coli* was also transformed with pESC-TRP, and plasmid DNA was prepared in the same manner (medium-scale extraction). PCR was performed with the prepared plasmid pME-Lyn as a template using following primers

a forward primer:

5'-cccggatcca tgggatgtat aaaatcaaaa-3' (SEQ ID NO: A-1), and

a reverse primer:

5'-catgtcgaca ggctgctgct ggtattgccc-3' (SEQ ID NO: A-2)

to amplify *lyn* gene. The resulting PCR product was treated with *Bam*HI and *Sal*I, and DNA was then collected by cut-out from the gel. As to pESC-TRP, after the treatment with *Bam*HI and *Sal*I, DNA was collected by cut-out from a gel. The ligation was carried out with *lyn* gene and a fragment of pESC-TRP collected by these experimental procedures, and *Escherichia coli* was transformed with the resulting product. After the resulting transformant was confirmed by a mini-prep, plasmid DNA was prepared (medium-scale extraction). A nucleotide sequence of the introduced gene was confirmed by sequencing from both sides of a gene-introduced portion.

(ii) Introduction of *lyn* Gene in YPH500 Strain (ρ^+) and YPH500-12 Strain (ρ^-)

An YPH500 strain (ρ^+) and an YPH500-12 Strain (ρ^-) were transformed with the prepared plasmid according to the lithium acetate method. The resulting transformant was subcultured to a different CSM-TRP solid medium, and cultured at 30°C for 2 to 3 days, and thereby a transformant having no tryptophan-requiring property was screened.

(10) Expression of *parp1* Gene in Wild-Type Strain and Respiration Ability-Deficient Strain of Yeast *S. cerevisiae*

In this example, as a vector, pESC-URA was used for subcloning. In addition, as *parp1* gene, total RNA carrying the gene (originated from WI38 cells) was amplified, and the resulting gene was used.

(i) Preparation of pESC-URA/PARP1

RT-PCR was performed with the total RNA collected from WI38 cells as a template using following primers

a forward primer:

5'-atggcggagt cttcggataa-3' (SEQ ID NO: A-3), and

a reverse primer:

5'-cccgtcgacc cacagggagg tcttaaaat-3' (SEQ ID NO: A-4)

to amplify *parp1* gene. The resulting PCR product was treated with *Sal*I, and DNA was then collected by cut-out from the gel. pESC-URA was treated with *Bam*HI, and then blunt-ended. The resulting product was treated with *Sal*I, and DNA was collected by cut-out from a gel. The ligation was carried out with *parp1* gene and a fragment of pESC-URA collected by these experimental procedures, and *Escherichia coli* was transformed with the resulting product. After the resulting transformant was confirmed by a mini-prep, plasmid DNA was prepared. A nucleotide sequence of the introduced gene was confirmed by sequencing from both sides of a gene-introduced portion. The constructed plasmid was named pESC-URA/PARP1.

(ii) Introduction of *parp1* Gene in YPH500 Strain (ρ^+) and YPH500-12 Strain (ρ^-)

An YPH500 strain (ρ^+) and an YPH500-12 Strain (ρ^-) were transformed with the prepared plasmid according to the lithium acetate method. The resulting transformant was subcultured to a different CSM-URA solid medium, and cultured at 30°C for 2 to 3 days, and thereby a transformant having no uracil-requiring property was screened.

4. Experimental Results

(1) Observation on Growth of Wild-Type Strain and Respiration Ability-Deficient Strain of Yeast *S. cerevisiae*

Since the wild-type yeast *S. cerevisiae* strain produces a red pigment, red colonies are formed in the YPD plate; on the other hand, the colonies of the respiration ability-deficient yeast *S. cerevisiae* strain lacks a red pigment, so that white colonies are formed.

Therefore, whether or not the YPH500 strain (ρ^+) and the YPH500-12 strain (ρ^-) formed red colonies and/or white colonies was observed by subjecting those strains to a single colony isolation on an YPD plate.

In other words, cells freeze-stored in a freezer at -80°C were scraped together with a platinum loop, and thinly spread over the YPD plate, so that the cells are separated from each other, thereby prompting the formation of independent colonies.

The growth of the YPH500 strain (ρ^+) is shown in Figure I, and YPH500-12 strain (ρ^-) is shown in Figure II.

FIG. I **Growth of the Wild-Type Strain**

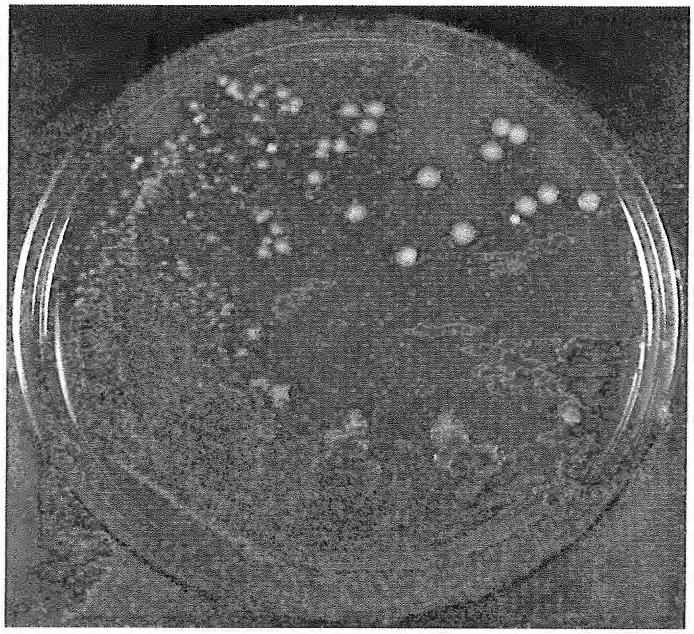
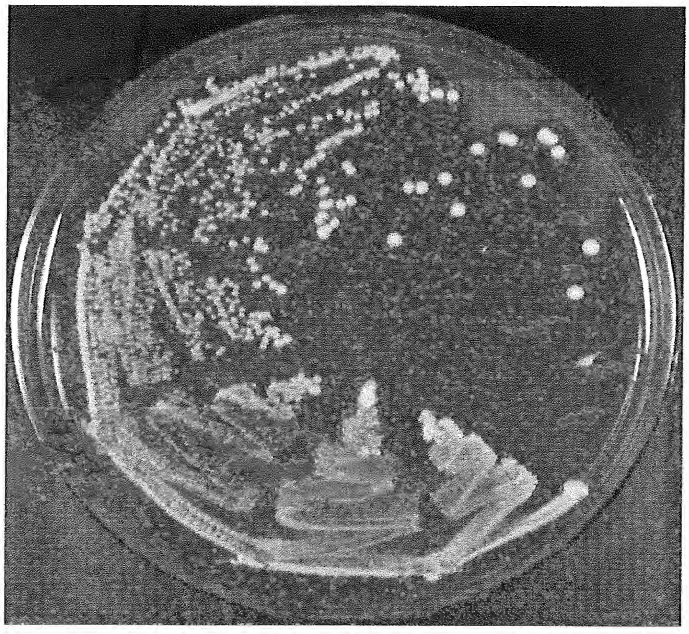


FIG. II **Growth of the Respiration Ability-Deficient Strain**



It can be seen from Figure I that the wild-type YPH500 strains form red colonies in the YPD plate, and at the same time form white colonies of smaller sizes, demonstrating that the YPH500 strains are undergoing mutation at a certain frequency, and consequently developing a respiration-deficient strain. On the other hand, it can be seen from Figure II that the respiration ability-deficient YPH500-12 strains only form white colonies, so that uniform size colonies are formed.

It was confirmed from the above results that the wild-type YPH500 strain was developed into two kinds of strains, a wild-type strain and a respiration ability-deficient strain, but the respiration ability-deficient YPH500-12 strain was not developed any more, showing uniform growth.

(2) Observation on Growth of YPH500 Strain (ρ^+) and YPH500-12 Strain (ρ^-) by Expression of Caf Protein

A pESC vector, which is a protein expression vector for the yeast, has a *GAL1* promoter, and expresses a desired gene only in the presence of galactose. Therefore, a transformed yeast was cultured in the presence of galactose to induce expression of Caf protein.

More specifically, regarding the *hcaf1* gene-introduced YPH500 strain (ρ^+) described in item (ii) of the section (8), colonies were inoculated from the plate into 5 mL of CSM-URA Glucose (2%) medium, and pre-cultured. The pre-culture was carried out at 30°C for 60 hours, and thereafter a 0.1 mL culture medium each was taken from the culture medium and subcultured to 5 mL of CSM-URA Galactose (2%) medium, and the growth was observed.

For comparison, the growth of the wild-type strain of the yeast (no

expression of Caf protein) was observed by cultivating a pESC-URA-introduced YPH500 strain (ρ^+) (not introduced with *hcaf1* gene) under the same conditions as above.

On the other hand, regarding the *hcaf1* gene-introduced YPH500-12 strain (ρ^-) described in item (ii) of the section (8), colonies were inoculated from the plate into 5 mL of a CSM-URA Glucose (2%) medium, and pre-pre-cultured. At a point where the colonies were pre-pre-cultured at 30°C for 162 hours, a 0.1 mL culture medium each was taken from the culture medium, and further pre-cultured for 122 hours, and a 0.1 mL culture medium each was taken from the culture medium and subcultured to 5 mL each of a CSM-URA Raffinose (2%) medium and a CSM-URA Raffinose(2%)+Galactose(2%) medium, and the growth was observed.

KU values for each of the YPH500 strain (ρ^+) and the YPH500-12 strain (ρ^-) were plotted against the culture time, and proliferation curves were prepared. The proliferation curves for the YPH500 strain (ρ^+) are shown in Figure III, and the proliferation curves for the YPH500-12 strain (ρ^-) are shown in Figure IV. Here, the data for the YPH500 strain and the YPH500-12 strain are shown in Tables A and B, respectively.

Table A Growth of the Wild-Type Strains in Non-Expressed State and Caf1 Expressed State

Time (hr)	0	22	26	28	32	34	46	48	50	52	54	56	58
YPH500 in Non-Expressed State													
1	7	8	8	14	24	29	102	114	123	131	137	140	150
2	9	11	12	16	22	31	84	98	106	114	123	127	134
3	7	8	9	12	15	20	54	58	75	85	100	107	125
4	7	8	8	13	16	20	69	81	93	107	110	118	130
5	4	6	7	9	13	13	45	49	61	72	80	87	95
6	10	15	18	23	36	44	127	135	143	148	157	157	161
Average	7.3	9.3	10.3	14.5	21.0	26.2	80.2	89.2	100.2	109.5	117.8	122.7	132.5
SD	2.1	3.2	4.1	4.8	8.5	10.9	30.8	33.0	30.4	28.2	27.3	24.6	22.8
CV (%)	28.2	34.3	40.0	32.9	40.4	41.8	38.4	37.0	30.3	25.8	23.2	20.1	17.2
YPH500 in Expressed State													
1	6	8	8	15	21	27	89	100	110	113	120	124	135
2	7	7	7	7	9	10	19	24	26	30	35	38	47
3	7	9	9	10	11	12	21	25	27	32	34	38	47
4	4	4	4	4	4	4	5	5	6	7	10	10	10
5	7	8	8	9	10	12	22	27	28	31	39	41	48
6	11	11	11	11	11	14	25	26	30	33	40	41	51
Average	7.0	7.8	7.8	9.3	11.0	13.2	30.2	34.5	37.8	41.0	46.3	48.7	56.3
SD	2.3	2.3	2.3	3.7	5.5	7.6	29.7	33.1	36.4	36.6	37.7	38.8	41.5
CV (%)	32.6	29.6	29.6	39.9	50.5	57.7	98.3	96.0	96.3	89.3	81.5	79.7	73.7

(Unit: KU)

Table B Growth of the Respiration Ability-Deficient Strains in Non-Expressed State and Caf1 Expressed State

Time (hr)	0	13	18	22	37	40	43	48
YPH500-12 in Non-Expressed State								
1	7	12	18	29	136	150	159	160
2	10	10	21	31	137	154	164	164
3	15	20	26	37	137	158	167	167
4	7	11	19	30	136	154	165	165
5	7	10	16	24	121	147	160	163
6	8	13	21	35	150	162	165	165
Average	9.0	12.7	20.2	31.0	136.2	154.2	163.3	164.0
SD	3.2	3.8	3.4	4.6	9.2	5.4	3.1	2.4
CV (%)	35.1	29.8	17.0	14.9	6.8	3.5	1.9	1.4
YPH500-12 in Expressed State								
1	9	15	22	26	58	65	80	103
2	11	16	23	27	55	64	75	97
3	8	12	20	25	53	62	73	96
4	9	14	23	28	59	68	81	104
5	12	17	24	26	51	56	65	85
6	17	22	28	33	63	70	83	107
Average	11.0	16.0	23.3	27.5	56.5	64.2	76.2	98.7
SD	3.3	3.4	2.7	2.9	4.4	4.9	6.6	7.9
CV (%)	29.9	21.3	11.4	10.5	7.7	7.7	8.7	8.0

(Unit: KU)

FIG. III Growth of the Wild-Type Strains in Non-Expressed State and Caf1 Expressed State

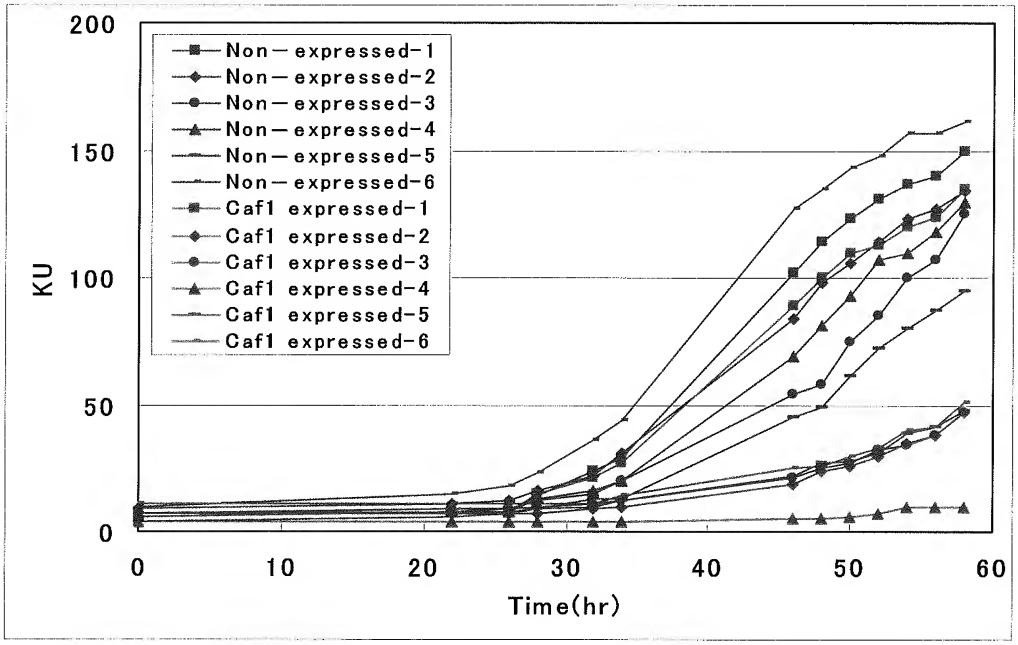
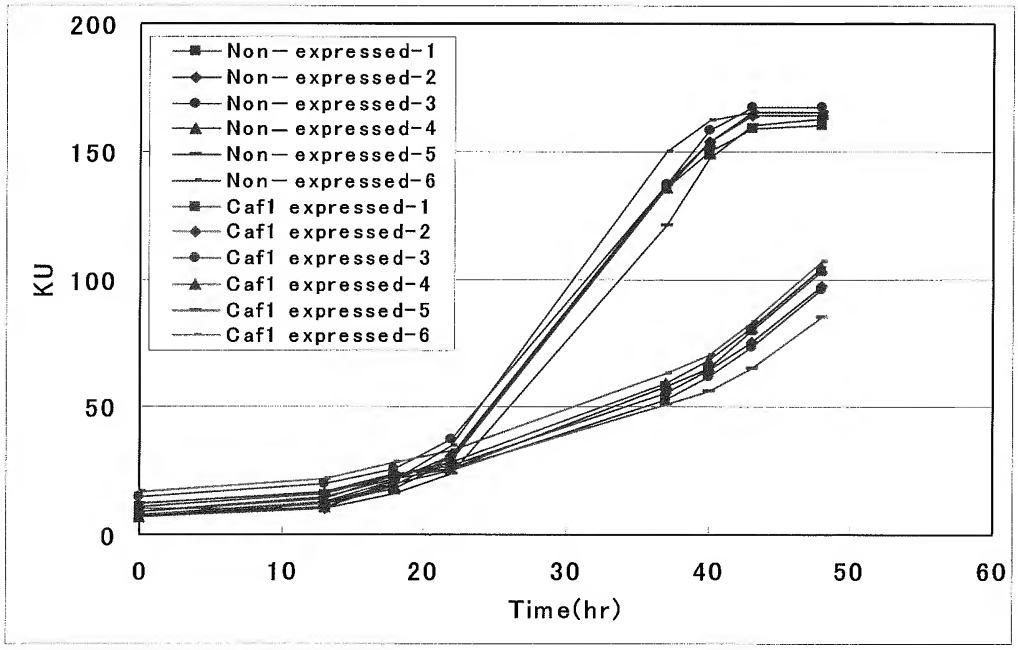


FIG. IV Growth of the Respiration Ability-Deficient Strains in Non-Expressed State and Caf1 Expressed State



It was found that both of the YPH500 strain and the YPH500-12 strain clearly showed reduced growth in the expressed state of the Caf protein, as compared to that of the non-expressed state. However, it was found from Figure III that the YPH500 strain (ρ^+) showed growth with variance both in a non-expressed state and in an expressed state of the Caf protein. On the other hand, it was found from Figure IV that the YPH500-12 strain (ρ^-) always showed uniform growth without variance for each of the transformed yeast in a non-expressed state and in an expressed state of the Caf protein.

It was confirmed from the above results that the respiration ability-deficient strain is more sensitive to the change in the growth state as compared to that of the wild-type strain, whereby showing more stable growth with smaller variance.

(3) Observation on Growth of YPH500 Strain (ρ^+) and YPH500-12 Strain (ρ^-) by Expression of Lyn Protein

Regarding the *lyn* gene-introduced YPH500 strain (ρ^+) described in item (ii) of the section (9), colonies were inoculated from the plate into 5 mL of CSM-TRP Glucose (2%) medium, and pre-cultured. The pre-culture was carried out at 30°C for 68 hours, and thereafter a 0.1 mL culture medium each was taken from the culture medium and subcultured to 5 mL of CSM-TRP Galactose (2%) medium, and the growth was observed.

For comparison, the growth of the wild-type strain of the yeast (no expression of Lyn protein) was observed by cultivating a pESC-TRP-introduced YPH500 strain (ρ^+) (not introduced with *lyn* gene) under the same conditions as above.

On the other hand, regarding the *lyn* gene-introduced YPH500-12 strain (ρ^-) described in item (ii) of the section (9), colonies were inoculated from the plate into 5 mL of a CSM-TRP Raffinose (2%) medium, and pre-cultured. At a point where the colonies were pre-cultured at 30°C for 89 hours, a 0.1 mL culture medium each was taken from the culture medium, and subcultured to 5 mL each of a CSM-TRP Raffinose (2%) medium and a CSM-TRP Raffinose(2%)+Galactose(2%) medium, and the growth was observed.

KU values for each of the YPH500 strain (ρ^+) and the YPH500-12 strain (ρ^-) were plotted against the culture time, and proliferation curves were prepared. The proliferation curves for the YPH500 strain (ρ^+) are shown in Figure V, and the proliferation curves for the YPH500-12 strain (ρ^-) are shown in Figure VI. Here, the data for the YPH500 strain and the YPH500-12 strain are shown in Tables C and D, respectively.

Table C Growth of the Wild-Type Strains in Non-Expressed State and Lyn Expressed State

Time (hr)	0	22	24	26	30	42	44	46	48	50	52	54	56
YPH500 in Non-Expressed State													
1	6	6	6	6	6	6	6	8	8	8	8	9	9
2	4	9	11	18	30	80	98	107	115	121	121	124	125
3	3	7	11	17	27	89	103	112	121	131	135	142	142
4	14	14	14	14	14	19	22	24	27	32	34	41	50
5	1	1	1	1	1	4	4	7	8	14	17	24	31
6	3	7	9	14	21	62	74	80	91	103	111	120	126
Average	5.2	7.3	8.7	11.7	16.5	43.3	51.2	56.3	61.7	68.2	71.0	76.7	80.5
SD	4.6	4.2	4.6	6.7	11.6	38.2	45.9	49.1	53.3	56.2	57.4	58.3	57.1
CV (%)	89.5	57.6	53.0	57.5	70.1	88.2	89.6	87.1	86.4	82.5	80.8	76.1	71.0
YPH500 in Expressed State													
1	3	3	3	3	3	3	3	5	5	6	6	7	7
2	3	3	3	3	3	4	4	4	4	4	4	5	5
3	5	5	5	5	5	9	10	14	14	16	17	19	20
4	7	7	7	7	7	7	8	10	10	10	11	12	12
5	5	5	5	5	7	13	14	17	20	25	27	33	34
6	6	6	6	6	6	12	13	14	16	22	25	25	26
Average	4.8	4.8	4.8	4.8	5.2	8.0	8.7	10.7	11.5	13.8	15.0	16.8	17.3
SD	1.6	1.6	1.6	1.6	1.8	4.1	4.5	5.3	6.3	8.6	9.7	10.9	11.4
CV (%)	33.1	33.1	33.1	33.1	35.5	51.2	52.5	49.5	54.9	62.1	64.4	64.7	65.6

(Unit: KU)

Table D Growth of the Respiration Ability-Deficient Strains in Non-Expressed State and Lyn Expressed State

Time (hr)	0	23	27	32	44	48	53	56
YPH500-12 in Non-Expressed State								
1	7	20	27	40	87	111	126	135
2	7	22	32	46	104	122	131	135
3	4	13	22	36	93	112	124	133
4	2	17	28	42	100	115	125	132
Average	5.0	18.0	27.3	41.0	96.0	115.0	126.5	133.8
SD	2.4	3.9	4.1	4.2	7.5	5.0	3.1	1.5
CV (%)	49.0	21.8	15.1	10.2	7.8	4.3	2.5	1.1
YPH500-12 in Expressed State								
1	10	18	18	23	40	47	56	64
2	8	12	16	18	33	38	47	52
3	7	13	20	22	44	54	67	77
4	6	14	18	20	35	40	53	60
Average	7.8	14.3	18.0	20.8	38.0	44.8	55.8	63.3
SD	1.7	2.6	1.6	2.2	5.0	7.3	8.4	10.4
CV (%)	22.0	18.5	9.1	10.7	13.1	16.3	15.0	16.5

(Unit: KU)

FIG. V Growth of the Wild-Type Strains in Non-Expressed State and Lyn Expressed State

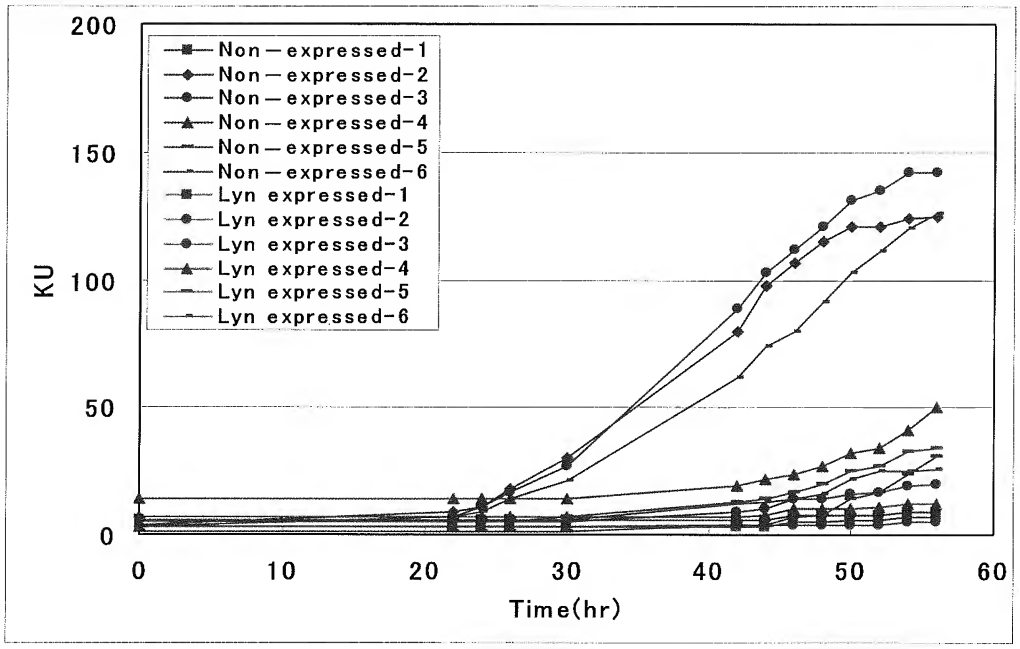
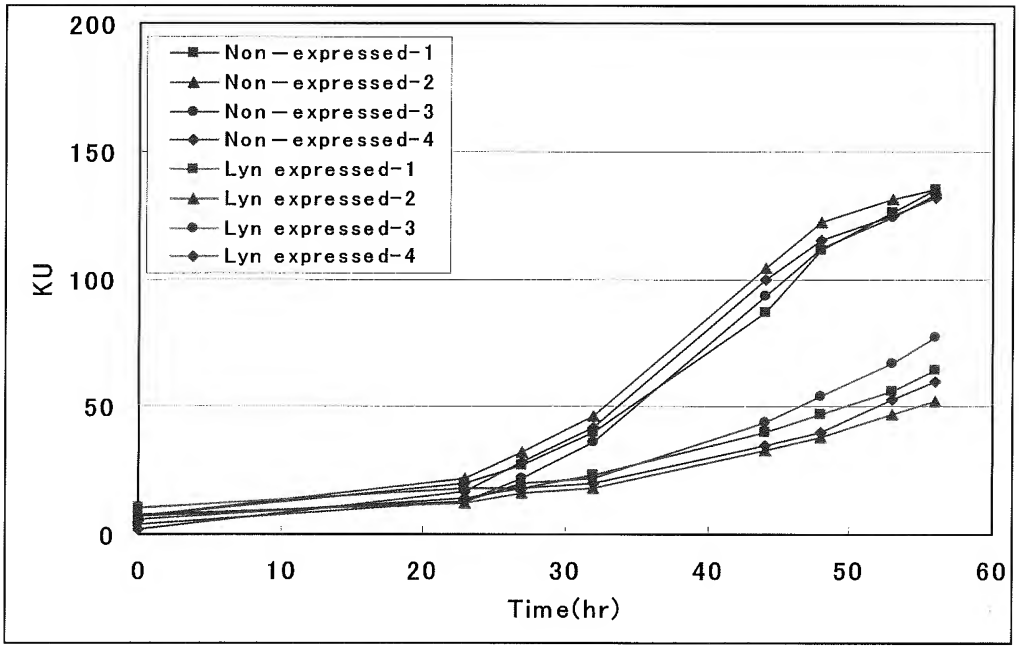


FIG. VI Growth of the Respiration Ability-Deficient Strains in Non-Expressed State and Lyn Expressed State



It was found that both of the YPH500 strain and the YPH500-12 strain clearly showed reduced growth in the expressed state of the Lyn protein, as compared to that of the non-expressed state. However, it was found from Figure V that the transformed YPH500 strains (ρ^+) showed growth with variance both in a non-expressed state and in an expressed state of the Lyn protein. On the other hand, it was found from Figure VI that the YPH500-12 strains (ρ^-) always showed uniform growth without variance for each of the transformed yeast in a non-expressed state and in an expressed state of the Lyn protein.

It was confirmed from the above results that the respiration ability-deficient strains showed more stable growth with smaller variance.

(4) Observation on Growth of YPH500 Strain (ρ^+) and YPH500-12 Strain (ρ^-) by Expression of PARP1 Protein

Regarding each of the *parp1* gene-introduced YPH500 strain (ρ^+) and the *parp1* gene-introduced YPH500-12 strain (ρ^-) described in item (ii) of the section (10), the transformant was inoculated from the plate to 3 mL of CSM-URA Glucose (2%), and pre-pre-cultured at 30°C. At a point where the pre-pre-culture was carried out for 30 hours, the OD_{600} value of the culture medium was determined, and the culture medium was diluted with a CSM-URA Raffinose (2%) medium, so that the culture medium had a cell density of OD_{600} = 0.5 calculated from the found value. Three milliliters of the diluted culture medium was transferred to a test tube, and pre-cultured at 30°C. At a point where the pre-culture was carried out for 36 hours, the OD_{600} value of the culture medium was determined, and the culture medium was diluted with a CSM-URA Raffinose (2%) + Galactose (2%) medium, so that the culture

medium had a cell density of OD_{600} = 0.4 calculated from the found value. The diluted culture medium was mixed with the same volume of CSM-URA Raffinose (2%) + Galactose (2%) medium with addition of 0 μM, 20 μM, 60 μM, or 200 μM NU1025, to prepare an NU1025-added cell suspension (cell density: OD_{600} = 0.2) in which a final concentration of NU1025 was 0 μM, 10 μM, 30 μM, or 100 μM. The prepared NU1025-added cell suspension was transferred to a 96 well microplate, and subjected to stationary culture at 30°C, and the growth was observed. The observation of the growth was carried out by determining an OD_{600} value using a microplate reader.

For comparison, the growth of the wild-type strain of the yeast and the respiration ability-deficient strain of the yeast not introduced with *parp1* gene was observed by cultivating the pESC-URA-introduced YPH500 and YPH500-12 strains (not introduced with *parp1* gene) under the same conditions as above.

The proliferation curves for the YPH500 strain (ρ^+) are shown in Figures VII and IX, and the proliferation curves for the YPH500-12 strain (ρ^-) are shown in Figures VIII and X. Here, the data for the growth rates of the transformed YPH500 strains and YPH500-12 strains (not introduced with *parp1* gene) in the presence or absence of the inhibitor NU1025 are shown in Tables E and F. Also, the data for the growth rates of the transformed YPH500 strains and YPH500-12 strains (introduced with *parp1* gene) in the presence or absence of the inhibitor NU1025 are shown in Tables G and H.

Table E Growth of the Wild-Type Strains in Non-Expressed State of PARP1 Protein in the Presence and Absence of NU1025

Strain	Inhibitor	Time (hr)	0	3	12	15	18	21	24	27	37
YPH500	0 μM	Ave. n=6	0.023	0.027	0.105	0.171	0.264	0.348	0.384	0.476	0.545
	10 μM	Ave. n=6	0.022	0.026	0.101	0.165	0.254	0.329	0.348	0.443	0.544
	30 μM	Ave. n=6	0.022	0.025	0.098	0.150	0.215	0.288	0.321	0.447	0.507
	100 μM	Ave. n=6	0.022	0.024	0.095	0.141	0.183	0.190	0.224	0.262	0.433
YPH500	0 μM	Ave. n=6	0.023	0.028	0.093	0.160	0.263	0.381	0.458	0.505	0.587
	10 μM	Ave. n=6	0.023	0.027	0.095	0.163	0.266	0.382	0.456	0.507	0.579
	30 μM	Ave. n=6	0.022	0.027	0.094	0.161	0.265	0.382	0.457	0.506	0.585
	100 μM	Ave. n=6	0.022	0.026	0.092	0.158	0.261	0.379	0.453	0.501	0.576
YPH500	0 μM	Ave. n=6	0.023	0.030	0.169	0.286	0.412	0.488	0.533	0.566	0.623
	10 μM	Ave. n=6	0.023	0.029	0.170	0.290	0.414	0.484	0.530	0.561	0.616
	30 μM	Ave. n=6	0.022	0.029	0.169	0.290	0.410	0.485	0.528	0.562	0.614
	100 μM	Ave. n=6	0.022	0.028	0.167	0.284	0.408	0.482	0.527	0.558	0.608
Whole	0 μM	Average	0.0230	0.0280	0.1224	0.2057	0.3129	0.4054	0.4583	0.5156	0.5849
		SD	0.0003	0.0016	0.0408	0.0698	0.0860	0.0730	0.0748	0.0461	0.0393
		CV (%)	1.3	5.7	33.4	33.9	27.5	18.0	16.3	8.9	6.7
	10 μM	Average	0.0226	0.0274	0.1217	0.2060	0.3113	0.3983	0.4444	0.5037	0.5798
		SD	0.0004	0.0015	0.0421	0.0726	0.0887	0.0791	0.0915	0.0589	0.0358
		CV (%)	1.7	5.5	34.6	35.2	28.5	19.8	20.6	11.7	6.2
	30 μM	Average	0.0223	0.0270	0.1201	0.2003	0.2967	0.3847	0.4352	0.5048	0.5686
		SD	0.0000	0.0018	0.0427	0.0779	0.1013	0.0985	0.1049	0.0575	0.0554
		CV (%)	0.0	6.8	35.5	38.9	34.1	25.6	24.1	11.4	9.7
	100 μM	Average	0.0218	0.0261	0.1176	0.1940	0.2839	0.3502	0.4012	0.4403	0.5388
		SD	0.0003	0.0017	0.0424	0.0783	0.1141	0.1482	0.1577	0.1574	0.0930
		CV (%)	1.3	6.7	36.0	40.3	40.2	42.3	39.3	35.7	17.3

Table F Growth of the Respiration Ability-Deficient Strains in Non-Expressed State of PARP1 Protein in the Presence and Absence of NU1025

Strain	Inhibitor	Time (hr)	0	3	12	15	18	21	24	27	37
YPH500-12	0 μM	Ave. n=6	0.027	0.025	0.057	0.082	0.123	0.178	0.257	0.362	0.652
	10 μM	Ave. n=6	0.027	0.025	0.055	0.081	0.124	0.181	0.258	0.368	0.649
	30 μM	Ave. n=6	0.027	0.025	0.053	0.083	0.124	0.182	0.263	0.373	0.650
	100 μM	Ave. n=6	0.026	0.024	0.051	0.082	0.123	0.178	0.256	0.364	0.647
YPH500-12	0 μM	Ave. n=6	0.025	0.026	0.055	0.081	0.121	0.178	0.256	0.359	0.639
	10 μM	Ave. n=6	0.024	0.026	0.054	0.079	0.120	0.178	0.257	0.360	0.636
	30 μM	Ave. n=6	0.024	0.026	0.052	0.079	0.121	0.179	0.258	0.365	0.635
	100 μM	Ave. n=6	0.023	0.024	0.051	0.078	0.120	0.177	0.255	0.357	0.633
YPH500-12	0 μM	Ave. n=6	0.025	0.026	0.054	0.079	0.117	0.172	0.246	0.348	0.636
	10 μM	Ave. n=6	0.031	0.025	0.053	0.077	0.117	0.171	0.246	0.349	0.632
	30 μM	Ave. n=6	0.034	0.026	0.053	0.079	0.119	0.173	0.250	0.355	0.632
	100 μM	Ave. n=6	0.035	0.025	0.052	0.078	0.119	0.172	0.248	0.351	0.636
Whole	0 μM	Average	0.0253	0.0256	0.0553	0.0807	0.1203	0.1758	0.2527	0.3563	0.6423
		SD	0.0010	0.0003	0.0013	0.0018	0.0027	0.0036	0.0061	0.0072	0.0085
		CV (%)	4.0	1.4	2.3	2.2	2.3	2.1	2.4	2.0	1.3
	10 μM	Average	0.0272	0.0252	0.0536	0.0790	0.1202	0.1767	0.2535	0.3589	0.6389
		SD	0.0037	0.0003	0.0008	0.0020	0.0034	0.0053	0.0067	0.0093	0.0088
		CV (%)	13.6	1.0	1.6	2.6	2.9	3.0	2.6	2.6	1.4
	30 μM	Average	0.0280	0.0252	0.0527	0.0802	0.1213	0.1779	0.2569	0.3641	0.6388
		SD	0.0051	0.0006	0.0008	0.0027	0.0024	0.0043	0.0067	0.0089	0.0093
		CV (%)	18.2	2.3	1.5	3.4	1.9	2.4	2.6	2.4	1.5
	100 μM	Average	0.0279	0.0242	0.0514	0.0793	0.1209	0.1756	0.2531	0.3574	0.6386
		SD	0.0058	0.0006	0.0009	0.0024	0.0021	0.0032	0.0042	0.0066	0.0072
		CV (%)	20.9	2.4	1.7	3.0	1.7	1.8	1.7	1.8	1.1

Table G Growth of the Wild-Type Strains in Expressed State of PARP1 Protein in the Presence and Absence of NU1025

Strain	Inhibitor	Time (hr)	0	3	12	15	18	21	24	27	37
YPH500	0 μM	Ave. n=6	0.024	0.026	0.041	0.051	0.063	0.079	0.095	0.106	0.117
	10 μM	Ave. n=6	0.023	0.025	0.047	0.061	0.082	0.108	0.139	0.165	0.230
	30 μM	Ave. n=6	0.023	0.025	0.049	0.066	0.091	0.119	0.159	0.202	0.301
	100 μM	Ave. n=6	0.024	0.027	0.050	0.069	0.096	0.129	0.174	0.224	0.364
YPH500	0 μM	Ave. n=6	0.027	0.028	0.049	0.065	0.083	0.107	0.133	0.164	0.327
	10 μM	Ave. n=6	0.025	0.027	0.052	0.070	0.094	0.121	0.158	0.204	0.388
	30 μM	Ave. n=6	0.026	0.027	0.053	0.074	0.101	0.134	0.178	0.235	0.417
	100 μM	Ave. n=6	0.027	0.029	0.055	0.076	0.106	0.142	0.192	0.253	0.439
YPH500	0 μM	Ave. n=6	0.027	0.018	0.050	0.078	0.109	0.147	0.190	0.228	0.266
	10 μM	Ave. n=6	0.025	0.023	0.084	0.130	0.187	0.259	0.315	0.334	0.383
	30 μM	Ave. n=6	0.026	0.015	0.065	0.111	0.189	0.299	0.363	0.398	0.461
	100 μM	Ave. n=6	0.027	0.010	0.060	0.109	0.205	0.312	0.374	0.409	0.474
Whole	0 μM	Average	0.0256	0.0238	0.0466	0.0646	0.0852	0.1107	0.1391	0.1658	0.2369
		SD	0.0018	0.0056	0.0046	0.0135	0.0232	0.0341	0.0478	0.0613	0.1080
		CV (%)	7.1	23.4	9.9	20.9	27.2	30.8	34.4	37.0	45.6
	10 μM	Average	0.0244	0.0247	0.0611	0.0870	0.1211	0.1626	0.2038	0.2346	0.3336
		SD	0.0013	0.0021	0.0200	0.0372	0.0577	0.0836	0.0963	0.0885	0.0898
		CV (%)	5.5	8.4	32.7	42.8	47.7	51.4	47.3	37.7	26.9
	30 μM	Average	0.0247	0.0222	0.0558	0.0836	0.1269	0.1837	0.2334	0.2783	0.3927
		SD	0.0016	0.0065	0.0084	0.0237	0.0543	0.1000	0.1127	0.1046	0.0828
		CV (%)	6.6	29.5	15.0	28.3	42.8	54.5	48.3	37.6	21.1
	100 μM	Average	0.0257	0.0220	0.0552	0.0846	0.1356	0.1940	0.2464	0.2954	0.4258
		SD	0.0014	0.0101	0.0051	0.0212	0.0602	0.1023	0.1105	0.0993	0.0561
		CV (%)	5.6	46.0	9.2	25.0	44.4	52.7	44.9	33.6	13.2

Table H Growth of the Respiration Ability-Deficient Strains in Expressed State of PARP1 Protein in the Presence and Absence of NU1025

Strain	Inhibitor	Time (hr)	0	3	12	15	18	21	24	27	37
YPH500-12	0 μM	Ave. n=6	0.026	0.024	0.029	0.033	0.039	0.044	0.051	0.059	0.086
	10 μM	Ave. n=6	0.025	0.024	0.037	0.046	0.059	0.074	0.088	0.104	0.180
	30 μM	Ave. n=6	0.025	0.023	0.040	0.052	0.072	0.091	0.112	0.138	0.240
	100 μM	Ave. n=6	0.026	0.025	0.044	0.060	0.081	0.105	0.135	0.168	0.315
YPH500-12	0 μM	Ave. n=6	0.024	0.023	0.030	0.033	0.039	0.045	0.053	0.060	0.081
	10 μM	Ave. n=6	0.024	0.024	0.036	0.045	0.057	0.070	0.084	0.100	0.167
	30 μM	Ave. n=6	0.023	0.023	0.040	0.052	0.069	0.087	0.107	0.130	0.228
	100 μM	Ave. n=6	0.024	0.025	0.042	0.057	0.078	0.101	0.129	0.161	0.297
YPH500-12	0 μM	Ave. n=6	0.032	0.024	0.030	0.034	0.038	0.043	0.048	0.058	0.075
	10 μM	Ave. n=6	0.030	0.024	0.037	0.045	0.056	0.071	0.086	0.104	0.172
	30 μM	Ave. n=6	0.027	0.024	0.040	0.051	0.070	0.089	0.109	0.136	0.237
	100 μM	Ave. n=6	0.025	0.024	0.042	0.055	0.077	0.101	0.130	0.165	0.303
Whole	0 μM	Average	0.0271	0.0237	0.0299	0.0331	0.0385	0.0441	0.0507	0.0592	0.0805
		SD	0.0041	0.0006	0.0005	0.0004	0.0004	0.0008	0.0024	0.0010	0.0054
		CV (%)	15.0	2.5	1.7	1.3	1.1	1.9	4.8	1.7	6.7
	10 μM	Average	0.0262	0.0239	0.0367	0.0452	0.0573	0.0716	0.0862	0.1027	0.1729
		SD	0.0034	0.0002	0.0006	0.0009	0.0013	0.0023	0.0020	0.0026	0.0065
		CV (%)	12.9	0.8	1.6	2.0	2.3	3.2	2.3	2.5	3.8
	30 μM	Average	0.0250	0.0233	0.0397	0.0517	0.0701	0.0887	0.1094	0.1344	0.2351
		SD	0.0020	0.0003	0.0003	0.0003	0.0016	0.0020	0.0025	0.0044	0.0067
		CV (%)	7.9	1.4	0.6	0.6	2.3	2.3	2.3	3.3	2.9
	100 μM	Average	0.0248	0.0244	0.0425	0.0571	0.0787	0.1023	0.1311	0.1648	0.3050
		SD	0.0011	0.0006	0.0011	0.0022	0.0019	0.0026	0.0037	0.0035	0.0095
		CV (%)	4.5	2.6	2.6	3.8	2.4	2.5	2.8	2.1	3.1

FIG. VII Growth of the Wild-Type Strains in Non-Expressed State of PARP1 Protein in the Presence and Absence of NU1025

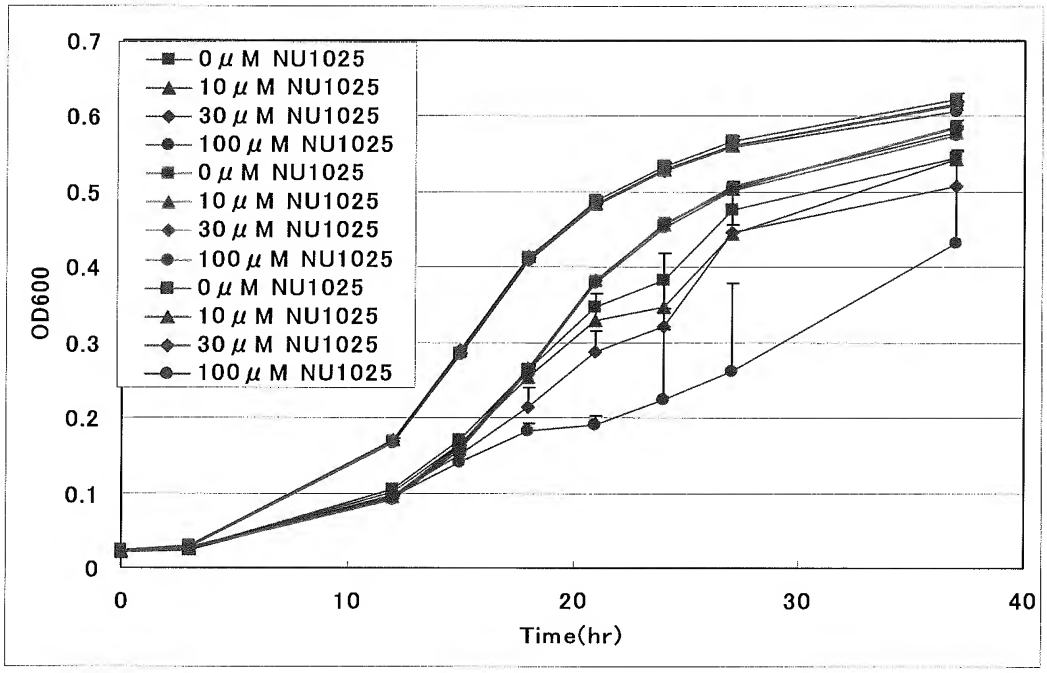


FIG. VIII Growth of the Respiration Ability-Deficient Strains in Non-Expressed State of PARP1 Protein in the Presence and Absence of NU1025

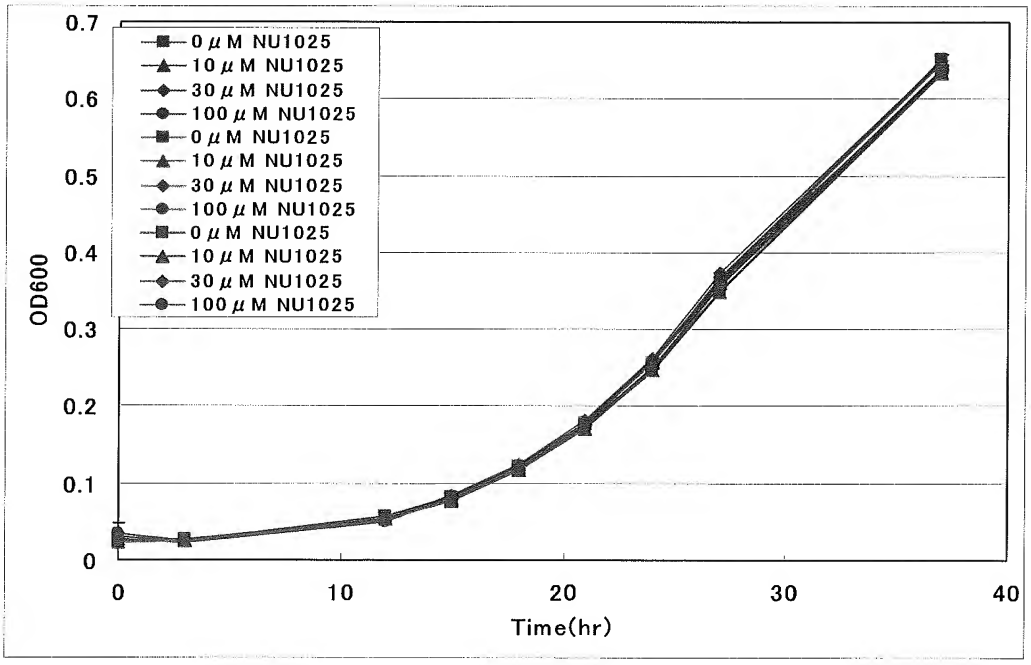


FIG. IX Growth of the Wild-Type Strains in Expressed State of PARP1 Protein in the Presence and Absence of NU1025

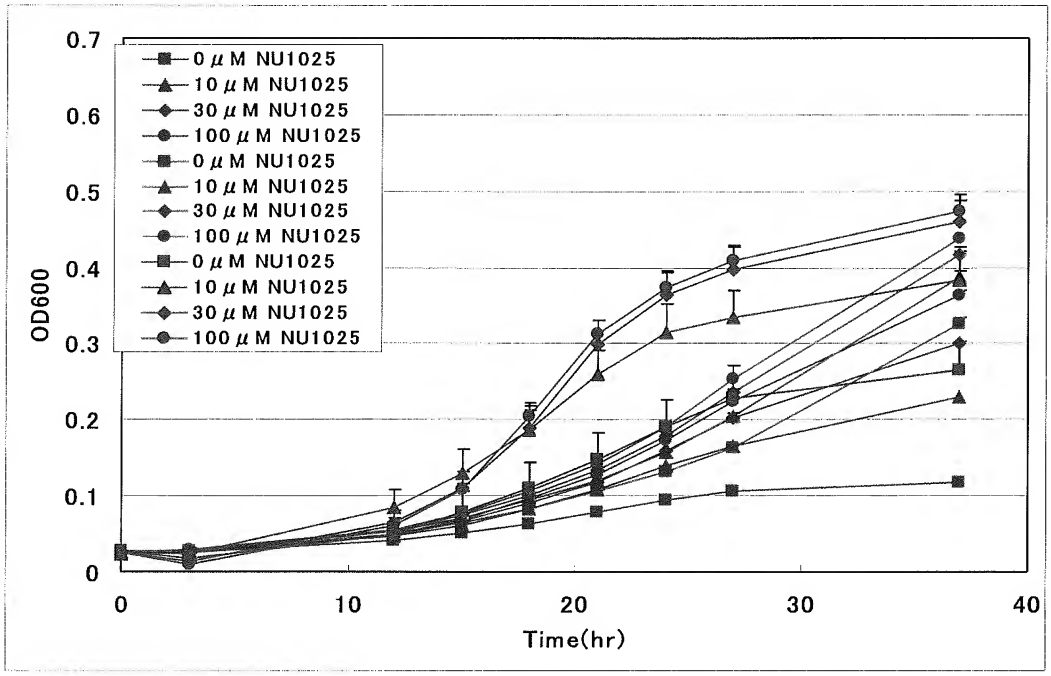
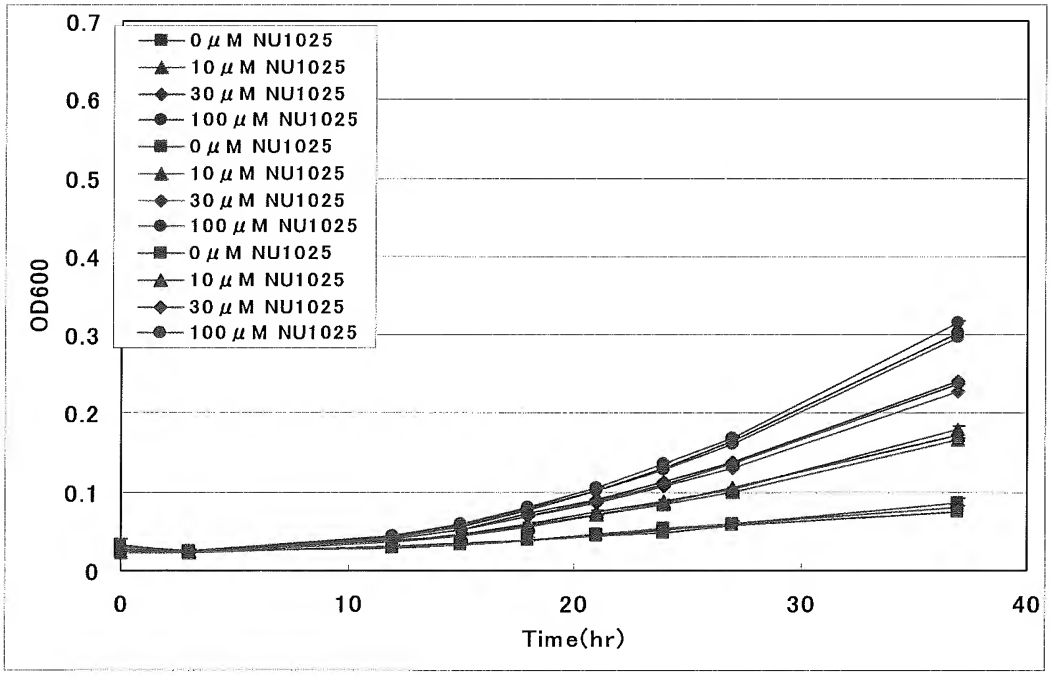


FIG. X Growth of the Respiration Ability-Deficient Strains in Expressed State of PARP1 Protein in the Presence and Absence of NU1025



It was found from Figures VII to X that the wild-type strains and the respiration ability-deficient strains clearly showed reduced growth in the expressed state of the PARP1 protein, as compared to that of the non-expressed state. In other words, when the growth of the wild-type strains in the medium without addition of NU1025 was compared, the lowering in the growth rate is found by PARP1 protein expression. This phenomenon was the same for the respiration ability-deficient strain.

In addition, in the wild-type strains and the respiration ability-deficient strains, it was found that the growth rate that was lowered by expression of the PARP1 protein recovered with the addition of NU1025 in a concentration-dependent manner. More specifically, in a case where 10 μM, 30 μM, or 100 μM NU1025 was added, the growth rate of the PARP1 protein expression strain increased with the increase in the concentration of the added NU1025.

However, at the same time, as shown in Figure IX and Table G, there is a large individual difference in growth between yeast transformants in the wild-type strains; more specifically, each of the transformants respectively showed different growth when the experiment was carried out using plural transformants that are different from each other. CV of the OD value at 27 hours showed the results exceeding 30%.

On the other hand, as shown in Figure X and Table H, almost no individual difference in growth between the yeast transformants was observed in the respiration ability-deficient strains. More specifically, when the experiment was carried out using plural transformants different from each other, the growth of the transformant showed uniform results. CV of the OD value at 27 hours was 4% or less.

It can be seen from the above results that transformants always maintaining the quality of a given growth rate can be obtained in the respiration ability-deficient strains, but such transformants cannot be secured in the wild-type strains. The respiration ability-deficient strains can be used as a reagent having stable quality, so that a determination can be enabled with a smaller variance.

5. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

6. Further declarant saith not.

Masao Tokunaga

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May. 16. 2007

Date

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